Kwang-Hyun Baek· Daniel Z. Skinner

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Kwang-Hyun Baek

Department of Crop and Soil Sciences, 210 Johnson Hall, Washington State University, Pullman, WA 99164-6420, USA

Daniel Z. Skinner ( )

USDA/ARS, Department of Crop and Soil Science, 209 Johnson Hall, Washington State University, Pullman, WA 99164-6420, USA

Corresponding author: D.Z. Skinner.

Tel.: +1-509-335-3475; fax: +1-509-335-2553

E-mail address: dzs@wsu.edu

Abstract

The sequences of the 3' untranslated region (UTR) of the manganese superoxide dismutase (MnSOD) genes in wheat (*Triticum aestivum*) were found to be quite variable with different predicted thermostabilities. The degradation rates of the 3' UTR variants and the coding region were measured following exposure to endogenous nucleases. The degradation rates of the 3' UTR variants for 15 min were not significantly different, meaning the degradation rates of the 3' UTR variants were not directly related to the thermostabilities. However, the degradation rate of the coding region was significantly faster than those of the 3' UTR variants. Further investigation revealed the coding region seemed to have specific sites for degradation, indicating a possibility of increasing MnSOD expression by the degradation site alteration.

Keywords Manganese superoxide dismutase · multigene family · Quantitative real time PCR · RNA secondary structure · RNA stability · Wheat

Abbreviations: cDNA, complementary DNA; MnSOD, mitochondrial manganese-superoxide dismutase; qRT-PCR, quantitative real time PCR; 3' UTR, 3' untranslated region

Introduction

Manganese superoxide dismutase (MnSOD; E.C.1.15.1.1) is an essential enzyme for the survival of aerobic life and serves to protect against oxidative stress in mitochondria (Carlioz and Touati 1986; Bowler et al. 1991; Møller 2001). MnSOD genes in eukaryotes are located in the nucleus, and encode a monomeric precursor protein with a mitochondria-targeting leader peptide (Halliwell and Gutteridge 1999). The precursor protein is targeted to the mitochondrial matrix, where it is processed to the active form after removal of the leader sequence and assembly of four monomeric units results in the active tetramer form (Bowler et al. 1989). The roles of MnSOD enzyme in plants have been studied extensively and found to confer tolerance to environmental stresses such as chilling, freezing, oxidative stress, and aluminum toxicity (Clare et al. 1984; McKersie et al. 1993; Breusegem et al. 1999;Yu et al. 1999; Basu et al. 2001).

Wheat (*Triticum aestivum*) is expected to have at least three copies of the MnSOD gene because wheat is an allohexaploid with three ancestral genomes. MnSOD genes in wheat comprise a multigene family, based on the alignment of the sequences of wheat MnSOD cDNAs at GenBank (accessions AF092524, U72212, and U73172) and of wheat cDNA clones isolated in our laboratory, genetic mapping data (Wu et al. 1999), and EST-mapping data at GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml). The members of the wheat MnSOD multigene family have slight differences in nucleotide and amino acid sequence, however, the final gene products, MnSOD enzyme, have the same enzymatic function. There is a high degree of sequence similarity among the genes (Wu et al. 1999) with relatively high variances in the 3' untranslated region (3' UTR) (Fig. 1).

Control of mRNA levels primarily depends on modulating the rate of mRNA synthesis; however, the levels of a given transcript can also be altered by post-transcriptional regulation (Khodursky and Bernstein 2003). Each step of the post-transcriptional pathway, including RNA processing, RNA transport, translation, and stability can be controlled in response to external or internal changes (Pesole et al. 2001; Stormo and Ji 2001). Although control of RNA stability is the least understood step, gene expression in almost all organisms is influenced by mRNA stability because the levels of RNA within the cytoplasm are regulated by the relative rates of RNA transcription versus degradation (Ross 1995; Abler and Green 1996; Khodursky and Bernstein 2003). The stabilities of some mRNAs in plants are known to change in response to many stimuli including hormones (Flores and Tobin 1988; Margossian et al. 1988), heat shock (Brodl and Ho 1992), sucrose starvation (Sheu et al. 1994), and light intensity (Dickey et al. 1992). The mRNA stability can be regulated by the sequence and the structural motifs of the 3' UTR (Newman et al. 1993; Rott et al. 1998 a,b; Komine et al. 2002); therefore, gene expression levels can be regulated by the 3' UTR sequence. Expression of many genes, particularly in chloroplasts, often is regulated at the post-transcriptional level and precise cleavage of motifs in the 3' UTR by endogenous ribonucleases and is a critical step in the regulatory process (Bollenbach and Stern 2003).

Our objectives were to determine whether wheat MnSOD mRNA transcripts are degraded by cleavage at specific sites by endogenous endoribonucleases, and to measure the impact of DNA sequence variation, and hence predicted secondary structure, on the stability of 3' UTRs exposed to endogenous ribonucleases.

Materials and Methods

Plant Growth and total RNA extraction

Seeds of winter wheat line 442 (Storlie et al. 1998) were planted in a commercial soil-less potting mix and grown for 6 days in a growth chamber maintained at 25 ^{o}C under 15 μmol quanta $m^{-2}s^{-1}$ cool white fluorescent light, 16 hr photoperiod. The secondary leaves were harvested, and one leaf was directly immersed in liquid nitrogen in a mortar. The leaves were left in the mortars for 0, 15, 30, 45, or 60 min after all liquid nitrogen in the mortar had evaporated. This step allowed degradation of the RNA by endogenous ribonucleases. Total RNA was extracted using Trizol reagent (Invitrogen, San Diego, CA, http://www.invitrogen.com) following the manufacturer's instructions. Briefly, liquid nitrogen was added to the mortar after the incubation time, and the leaves were ground using a pestle. One ml of Trizol solution was added to the mortar before thawing of the plant material, and total RNA was extracted according to manufacturer's instructions. Extracted total RNA was quantified with a spectrophotometer (Shimadzu UV-1601, Columbia, MD, http://www.shimadzu.com). The total RNA was stored at – 80 ^{o}C.

cDNA synthesis

Complementary DNA (cDNA) was synthesized using oligo dT primers and Thermoscript kit (Invitrogen, CA, San Diego, http://www.invitrogen.com). Gene-specific primers (forward: 5'-CGTCCGCCGTCGTCCA and reverse: 5'-AACAGCACTAGCGAACGAGTT) were used to

amplify the MnSOD gene using the cDNA template and Taq DNA polymerase. The PCR solution consisted of 1 X reaction buffer and 2 units of Taq polymerase from Promega (Madison, WI, http://www.promega.com), 2.0 mM $MgCl_2$, 100 nM primers, and cDNA made from 200 ng total RNA. The 25 μl reactions were covered with 20 μl mineral oil and amplified with a PCR profile of 3 min denaturation at 94°C, then 32 cycles of 30 s at 94°C, 40 sec at 52 °C, and 1.5 min at 72°C; then 5 min at 72°C. The PCR products were analyzed on 1.2% agarose gel, purified with GeneClean II kit (Q-Biogene, Carlsbad, CA, http://www.qbiogene.com), cloned into Topo-XL PCR vector (Invitrogen, San-Diego, CA, http://www.invitrogen.com), and sequenced at the DNA sequencing facility at Washington State University, Pullman, to confirm the cloned fragments were indeed parts of MnSOD genes. These clones were used as probes on Northern blots described below.

Three wheat MnSOD cDNA sequences from GenBank (accessions AF092524:MnSOD, U72212:MnSOD3.1, and U73172:MnSOD3.2) and three isolated by cDNA synthesis identified as K49-BA, K52-AA, and K56-AB were aligned using MultAlin program (Corpet, 1998; http://prodes.toulouse.inra.fr/multalin/multalin.html).

Quantititive real time PCR (qRT-PCR) measurement of degradation of MnSOD mRNA and Northern blot analysis

A RotorGene 2000 unit (Corbett Research, Sydney, Australia, http://www.corbettresearch.com) was used for qRT-PCR using SYBR green (product number S7567; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for detection of the product at the end of each amplification cycle (Bustin 2000; Karsai et al. 2002). The primers 58 A (5'-CAGAGGGTGCTGCTTTACAA)

and B (5'-GGTCACAAGAGGGTCCTGAT) used for detecting the middle of the MnSOD transcripts, were designed using Primer3 program (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The HPLC-purified primers (Operon Technologies, Inc., Alameda, CA, http://www.operon.com) used for detecting 3' UTR variants were designed manually to end in regions of different sequence in the 3' UTR variants.

Complementary DNA synthesis and qRT-PCR was carried out with Superscript One-step RT-PCR Platinum Taq kit (Invitrogen, San Diego, CA, http://www.invitrogen.com), with which the cDNA synthesis and PCR amplification were performed in a single tube. The buffer consisted of 1 X reaction buffer from the Superscript One-step RT-PCR kit, 2mM $MgCl_2$, 1 μM primers, 1:40,000 SYBR Green, and 125 ng total RNA in 20 μl reactions. The process consisted of cDNA synthesis for 15 min at 50°C followed by a PCR profile of a 3 min denaturation at 95°C, then 32 cycles of 20 s at 95°C, 30 s at 54°C, and 30 s at 72°C. The reaction solution was covered with 7 μl light mineral oil (Sigma, St. Louis, MO, http://www.sigmaaldrich.com) to prevent evaporation during qRT-PCR. All qRT-PCRs were repeated three times as completely independent replications. MnSOD transcripts were quantified in RNA isolated 0-60 min after thawing, at 15 min intervals. Known concentrations of cloned MnSOD double-stranded DNA were used to generate data for a standard curve. Melting curves of the final qRT-PCR products were generated to confirm a single PCR product had been formed.

Standard ^{32}P labeling and Northern blot hybridization protocols (Sambrook et al., 1989), using the qRT-PCR product from primers 58A and B (107 bp amplicon size) were used to assess

longevity of the MnSOD RNA transcripts. Eight μg of the total RNA were loaded on a 1 % denaturing gel (50 % formamide, 2.2 M formaldehyde, and 0.5 x MOPS buffer) provided with the instructions of Hybond N nylon membrane (Amersham Pharmacia Biotech, UK, http://www.amersham.com), observed with ethidium bromide stain and transmitted UV light (Sambrook et al. 1989), and transferred to Hybond N nylon membrane using a Turboblotter apparatus (Schleicher and Schuell, Keene, NH, http://www.schleicher-schuell.com). The nylon membranes were prehybridized at 65 ^{o}C for 1 hr in 10 ml of hybridization buffer consisting of 0.5 M sodium phosphate (pH 7), 7 % SDS, and 1 mM EDTA. Hybridization was carried out at 65 ^{o}C for 16 hr. Membranes were then washed twice in 2x SSC, 0.1 % (w/v) SDS for 5 min at room temperature; twice in 1x SSC, 0.1 % (w/v) SDS for 10 min at room temperature and; four times in 0.1x SSC, 0.1 % (w/v) SDS for 5 min at 65 ^{o}C as described in the manual of the Hybond N nylon membrane. Visualization of the radioactivity was performed with a phosphorimager 445 SI (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com).

Results

The 3' UTR variants of the wheat MnSOD genes were designated as AA (bases 728-730 present, and bases 768-783 present; MnSOD-AA and K52-AA, Fig. 1), AB (bases 728-730 present, and bases 768-783 absent; MnSOD3.2-AB and K56-AB, Fig. 1), BA (bases 728-730 absent, and bases 768-783 present; MnSOD3.1-BA and K49-BA, Fig. 1), and BB (bases 728-730 absent, and bases 768-783 absent; not found). The HPLC-purified primer sets (Table 1) were tested using cloned genes (K49, K53, and K55 in Fig. 1) as templates. The primers discriminated the plasmids very efficiently and the melting curves showed only a single fragment was amplified

(Fig. 2). Therefore, qRT-PCR with these primer sets discriminated between the 3' UTR variant forms and provided quantification of transcript copy numbers used to measure degradation dynamics of each form.

Total RNA was degraded by endogenous ribonucleases apparently liberated by freezing the plant tissue in liquid nitrogen followed by thawing at room temperature (Fig. 2). The ribosomal RNA bands were no longer visible after 15 min (Fig. 2). The thermostabilities of the secondary structure of the three 3' UTR variants we studied here were calculated with online software (Brodsky et al., 1992; Brodsky et al., 1995; http://www.genebee.msu.su/services/rna2_reduced.html). The thermostability energy was calculated as -35.6, -30, and -25.1 kcal/mol for K49-BA, K52-AA, and K56-AB, respectively (Table 2). The qRT-PCR profile revealed RNA of the 3' UTR variant forms AA, AB, and BA (Fig. 1) was expressed in plants grown at 20°C (Fig. 3). The data graphed in Fig. 3 indicated the AB form occurred with a 1.3-fold greater number of molecules (approximately 38.7 $x10^3$ per nanogram of total RNA) than AA form (approximately 30.3 $x10^3$) and a 2.1-fold greater number of molecules than AB form (approximately 18.2 $x10^3$). The calculated thermostabilities (Table 2) were inversely proportional to the degradation rates, which were 708, 1277, and 1410 molecules per min over 15 min and 561, 958, and 1150 molecules per min over 30 min for K49-BA, K52-AA, and K56-AB, respectively (Table 3). However, analysis of the percentage consumed after 15 min degradation showed there were no statistically significant differences in the 3' UTR variants (Table 3). The degradation in the middle part of the MnSOD gene, and therefore a measurement of all forms of the gene, was much more rapid at 5689 molecules per min over 15 min and 3104 molecules per min over 30 min. The degradation rates of these 3' UTR variants

and the coding region of MnSOD very closely followed exponential regression models (Table 3). Northern blot analysis (Fig. 4) indicated that the part of the coding region spanned by the probe we used (bases 455 to 561) had specific sites that were preferentially cut by the endogenous ribonucleases, indicated by the disappearance of the full-length fragment concommitant with appearance of two fragments of discrete size (Fig. 4). The larger fragment was progressively converted to the smaller fragment with advancing time (Fig. 4).

Discussion

A broad range of mRNA stabilities ranging from several minutes to several days have been reported in higher plants (Abler and Green, 1996). The 3' UTR variants in the wheat MnSOD multigene family occurred in significantly different concentrations during growth at 20°C and had different predicted secondary structure thermostability energies, but the percentages of the three transcript forms we examined degraded after 15 min of exposure to endogenous ribonucleases were not significantly different. Other forms are known to occur in wheat (Fig. 1), hence it is possible there are forms that degrade at different rates on a percentage basis. It has been reported that, in some cases, the thermostability of the RNA secondary structure is not the primary determinant of stability (Chen and Dill, 2000). Furthermore, several sequence motifs in the 3' UTR have been implicated in post-transcriptional regulation (Abler and Green, 1996). Although the degradation rates of the three 3' UTR structures we examined were not significantly different, all of the 3' UTR regions were more resistant to degradation than the middle part of wheat MnSOD transcript through 15 min. exposure to endogenous ribonucleases (Table 3).

The degradation of MnSOD mRNA appeared to be initiated within the coding region by endoribonucleases and proceeded rapidly in the middle of the gene resulting in fragments of a discrete size (Fig. 4). Endonucleolytic cleavages within the coding region of mRNAs has been reported previously (Parker and Jacobson 1990; Klaff 1995). This result suggested that endogenous endoribonucleases degraded the MnSOD transcript by scission of specific sites. Within 60 minutes of thawing, there appeared to be very little exoribonuclease activity within the region we examined. This result is consistent with the observation that mRNA transcripts are protected from degradation by exoribonuclease by 5' capping and 3' poly A structures (Prescott 1988). The approach of freezing in liquid nitrogen and allowing the tissue to thaw apparently was minimally destructive of these structures, and thereby allowed the activity of endogenous site-specific endoribonucleases to be observed. This method is an alternative to the application of mRNA synthesis inhibitors such as actinomycin D (Ohme-Takagi et al. 1993; Johnson et al. 2000) or cordycepin (Gutierrez et al. 2002). In our experience, the application of these inhibitors to wheat leaf tissue gave inconsistent results (data not shown), probably due to inconsistent penetration into the cells of the inhibitors. It is known that extraction from plant tissue frozen in liquid nitrogen results in efficient extraction of RNA (Ausubel et al., 1993; Duarte et al. 1999) and ribonucleases (Booker, 2004). Our data demonstrated that using liquid nitrogen for freezing and thawing for specific time intervals at room temperature or at controlled temperature can be a powerful tool for studying mRNA stability.

There have been many attempts to increase transcript levels in plant species (reviewed in Liang and Skinner, 2004), especially by regulating the promoter sequences. In general, the amount of a particular protein product is proportional to the amount of mRNA encoding that protein,

therefore increasing stability of a particular mRNA may result in increased expression of the protein. The mRNA degradation data we have presented here suggests there are “hot spots” for degradation by endogenous ribonucleases. These regions possibly could be engineered to be more resistant, leading to increased half-life and more protein production.

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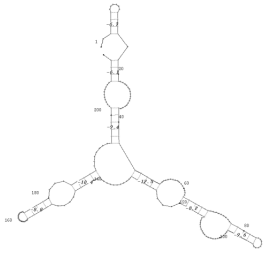
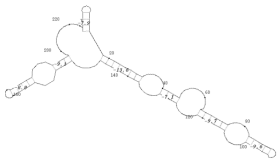
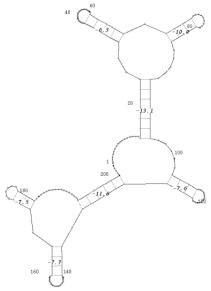
Table 1. DNA sequences of primers used for quantitative PCR determination of the concentrations of MnSOD transcripts in the coding region (base 455 to 561) or 3'UTR regions of wheat MnSOD sequence variants.

	Polymorphism			
Name	Bases 728-730	Bases 768-783	Forward primer (5'–3')[a]	Reverse primer (5'–3')[a]
Coding region	NA	NA	CAGAGGGTGCTGCTTTACAA	GGTCACAAGAGGGTCCTGAT
AA	present	present	GCRTGATTTGTCYGATGA	TTTTATTACTGCATRTACAAAGAT
AB	present	absent	GCRTGATTTGTCYGATGA	TTTATTACTGCATRTACAAACTC
BA	absent	present	GCRTGATTTGTCYGAGTA	TTTTATTACTGCATRTACAAAGAT

[a] Degenerate base composition was designated as R=A/G, Y=C/T.

Table 2. 3’ UTR structure thermostability and most stable secondary structure prediction[a]

Name	K49-BA[b]	K52-AA[b]	K56-AB[b]
Forms	-/+	+/+	+/-
Stability energy (kcal/mol)	-35.6	-30	-25.1
Secondary Structure of 3’UTR			

[a] The 3’ UTR regions from clone K49-BA, K52-AA, and K56-AB were used to calculate the thermostability and secondary structure prediction by an RNA secondary structure prediction program (http://www.genebee.msu.su/services/rna2_reduced.html).

[b] K52-AA form had bases 728-730 present and base 766-783 present, K56-AB had base 728-730 present and base 766-783 absent, and K49-BA form had base 728-730 absent and base 766-783 present. Nucleotide base numbering is according to the full-length sequence shown in Fig. 1.

Table 3. Comparison of degradation[a] rates of a coding region and 3' UTR variants of MnSOD

			Time			
			15 min		30 min	
Region[b]	Regression equation[c]	R^2	Degradation rate[d]	% Consumed[e]	Degradation rate[d]	% Consumed
Coding region	$y=91146x^{-2.958}$	0.999	5688	88.5 b	3104	96.6
AA	$y=22543x^{-2.689}$	0.987	1277	63.1 a	958	94.7
AB	$y=32530x^{-1.886}$	0.999	1410	54.7 a	1150	89.1
BA	$y=17378x^{-2.440}$	0.993	708	58.2 a	561	92.3

[a]Degradation of wheat MnSOD due to endogenous ribonucleases was measured in leaves allowed to thaw at room temperature for 0-60 min after freezing in liquid nitrogen.

[b]Regions AA, AB, and BA are 3' UTR regions with or without the nucleotide "gap" regions indicated in Figure 1. Form AA had bases 728-730 present and bases 766-783 present, AB had bases 728-730 present and bases 766-783 absent, and BA form had bases 728-730 absent and bases 766-783 present.

[c]Degradation was modeled using exponential regression of transcript copy number vs. time over 60 min of degradation. Regression analysis was carried out using a Microsoft® Excel spreadsheet and "Power" regression.

[d]Degradation rates were based on data graphed in Fig. 4 expressed as molecules per minute.

[e] % consumed within a column followed by the same letter were not significantly different according to Duncan's multiple range test, P=0.05.

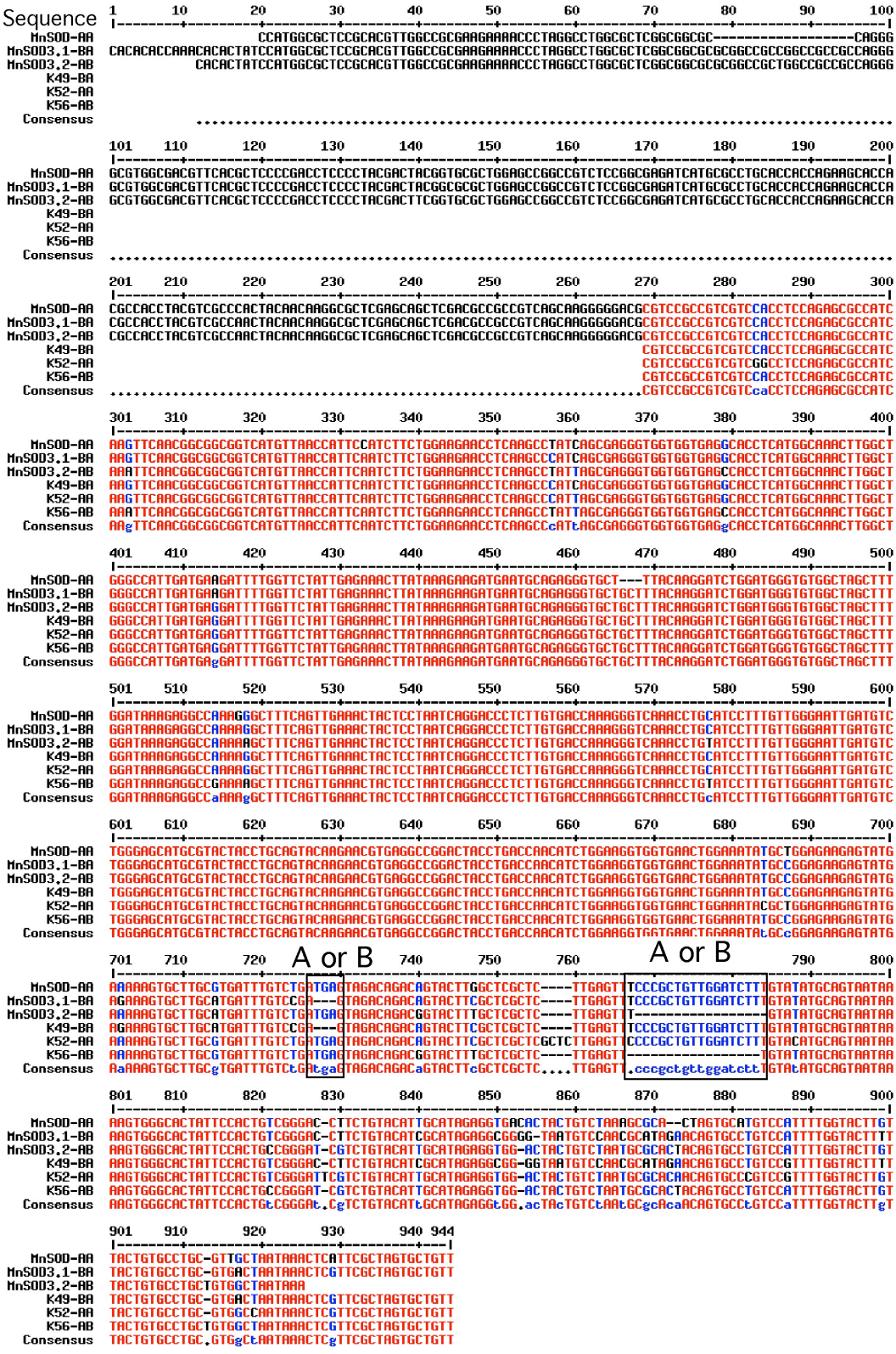
Figure Headings

Fig. 1. Alignment of cDNA sequences of wheat MnSOD genes. Forms with bases TGA at position 728 -730 and bases CCCGCTGTTCCATCTTT at position 768-783 are designated AA form. Forms without bases TGA at position 728 -730 seen in AA forms were designated BA form. Forms without bases CCCGCTGTTCCATCTTT at position 768-783 are designated as AB form. Genbank accession numbers of MnSOD-AA, MnSOD3.1-BA, and MnSOD3.2-AB were AF092524, U72212, and U73172, respectively. Clones K49-BA, K52-AA, and K56-AB were cloned and sequenced as part of this study. Sequences were aligned using MultAlign software (http://prodes.toulouse.inra.fr/multalin/multalin.html).

Fig 2. Denaturing gel to assess quality of RNA extracted from wheat leaves. Lanes 1, 2, 3, 4, and 5 were loaded with total RNA extracted from leaves frozen in liquid nitrogen and then thawed at room temperature for 0, 15, 30, 45, and 60 min, respectively. Lane 6 was loaded with 1 kb plus DNA ladder (Invitrogen, San Diego, CA).

Fig 3. qRT-PCR detection of the numbers of copies of wheat MnSOD mRNA extracted from leaves frozen in liquid nitrogen and then thawed for 0 to 60 minutes. The coding region was assayed as bases 455 to 561. Form AA had bases 728-730 present and bases 766-783 present, AB had bases 728-730 present and bases 766-783 absent, and BA form had bases 728-730 absent and bases 766-783 present. Nucleotide base numbering is according to the full-length sequence shown in Fig. 1.

Fig. 4. Northern Blot for detection of MnSOD genes. Lanes 1, 2, 3, 4, and 5 were loaded with total RNA extracted from leaves frozen in liquid nitrogen and then thawed at room temperature for 0, 15, 30, 45, and 60 min, respectively. Lane 6 was loaded with 1 kb plus DNA ladder (Invitrogen, San Diego, CA). hence, no signal was detected. This Northern blot was generated from the gel shown in Fig. 2.


Sequence
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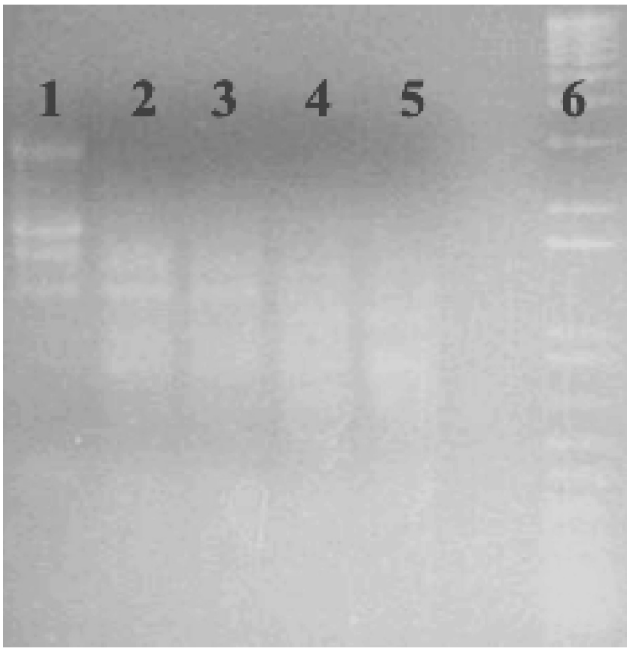
MnSOD3.1-BA
MnSOD3.2-AB
K49-BA
K52-AA
K56-AB
Consensus
A or B
A or B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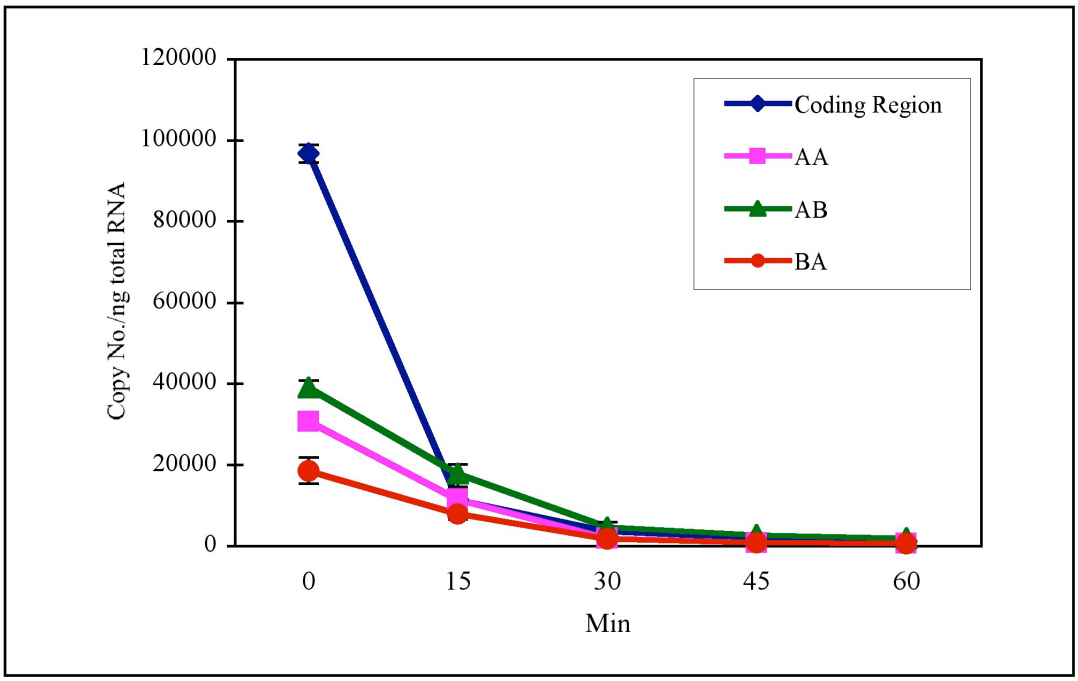
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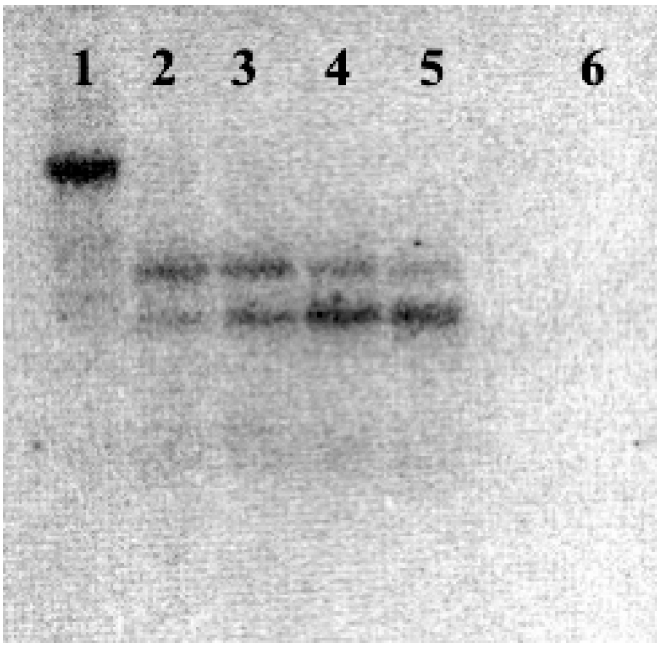
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